SYNOPSIS

hypothyroidism, pernicious anemia etc. (Castanet and Ortonne 1997; Kemp et al 2001). Antibodies to melanocyte antigens are present in circulation of most of the vitiliginous patients (Naughton et al 1983; Norris et al 1988; Cui et al 1992).

Most of the population studies on vitiligo are reported in European Americans, however clinical studies on Indian population are scarce. Since the environmental factors could account for the origin of autoimmunity/reactive oxygen species (ROS) in the susceptible patients, clinical studies on vitiligo in Indian patients addressing the different hypotheses is required. A systematic study was attempted by addressing the three main hypotheses and genetic association of *catalase* exon 9 T/C and *glutathione peroxidase* I codon 200 C/T single nucleotide polymorphisms in the vitiliginous patients to understand this disorder in Gujarat.

Objectives of the present study

1. To study the clinical profiles of vitiligo patients in Gujarat.
2. Evaluation of oxidative stress hypothesis in Gujarat vitiligo patients compared to controls.
3. Genetic association of *catalase* exon 9 T/C and *glutathione peroxidase* I codon 200 (C/T) SNP (single nucleotide polymorphism) in Gujarat vitiligo patients compared to controls.
4. Estimation of the protein levels of erythrocyte superoxide dismutase 1 in vitiligo patients compared to controls.
5. Evaluation of autoimmune hypothesis in Gujarat vitiligo patients compared to controls.
6. Evaluation of oxidative stress and autoimmune hypotheses at the onset of vitiligo.
7. Evaluation of neurochemical hypothesis in Gujarat vitiligo patients compared to controls.

Studies on clinical profiles of vitiligo in Gujarat:

The study consisted of 424 vitiligo patients. Clinical and demographic details of all the patients were obtained from the vitiligo clinical proformas. Detailed case history of 424 vitiligo patients was taken with the help of questionnaire to know the age of onset, duration of the disease, site of onset, types of vitiligo, precipitating factor/s and also family history of vitiligo.

Out of 424 outpatients, males constituted 38.44 % and females were 61.56%. Mean age of the patients was 25.59 years. The sites of onset were the lower limb, face, trunk, upper limb, genital, hand, labial and scalp in the descending order of frequency. Koebner phenomenon was observed in 12.74%, diabetes mellitus in 1.18%, leukotrichia in 9.2% and premature graying of hair in 23.11% patients. A family history of vitiligo was present in 21.93 % of the patients. Vitiligo vulgaris was the most common form of the disease, which constituted 52.36% of the patients followed by focal vitiligo (28.54%), segmental vitiligo (6.84%), acrofacial (7.55%), mucosal (2.83%) and universal vitiligo (1.89%).

Evaluation of oxidative stress hypothesis in Gujarat vitiligo patients compared to controls:

Evaluation of systemic oxidative stress in different age groups of vitiligo patients

This study was attempted to find out whether age has any relevance for the onset of the disease in Gujarat vitiligo patients. 124 vitiligo patients who had no associated diseases and 126 age matched healthy consenting volunteers as controls were used for this study. Blood was collected from the vitiligo patients after written informed consent had been obtained. Vitiligo patients were divided into five age groups i.e. 5 – 15 yrs, 16 – 25 yrs, 26 – 35 yrs, 36 – 45, 46-55 yrs for the analysis of the antioxidant status. Antioxidant enzymes in blood such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S transferase (GST), glutathione reductase (GR)

and non-enzymatic antioxidants such as reduced glutathione (GSH) and plasma vitamin E were estimated. Lipid peroxidation levels (LPO), the index of oxidative stress in erythrocytes and the reducing equivalent system, i.e. glucose 6 phosphate dehydrogenase (G6PDH) were also estimated. All age groups and overall vitiligo patients showed significant increase in erythrocyte superoxide dismutase activity compared to the age matched controls. Similarly a significant increase in the lipid peroxidation levels of vitiligo patients was observed in all age groups and overall vitiligo patients compared to their respective controls. A significant decline in glucose 6-phosphate dehydrogenase activity was also observed in all the age groups and overall vitiligo patients compared to controls. However, a significant decrease in glutathione peroxidase activity was observed in 5-15 yrs and 16-25 yrs age groups and overall vitiligo patients; other age groups (26-35 yrs, 36-45 yrs and 46-55 yrs) did not show significant change in GPX activity compared to controls. Erythrocyte catalase activity showed a significant decrease in overall vitiligo patients compared to controls; age groups 16-25 yrs and 36-45 yrs also showed significant decrease in catalase activity compared to respective controls; however, 5-15 yrs, 26-35 yrs and 46-55 yrs age groups did not show significant change in catalase activity compared to controls. A significant decrease in overall reduced glutathione levels was observed in vitiligo patients as well as in 36-45 yrs and 46-55 yrs age groups; however, no significant change was observed in other age groups (5-15 yrs, 16-25 yrs and 26-35 yrs). Plasma vitamin E levels, GST and GR showed no significant change in all age groups of vitiligo patients as well as overall vitiligo patients compared to controls.

Thus the impairment in systemic antioxidant system is observed in this study indicating that melanocyte damage in vitiligo patients may be linked with generalized oxidative stress. Significant increase in lipid peroxidation levels suggest that systemic oxidative stress is present in all age groups of vitiligo patients compared to their respective controls. Increased SOD activity

in vitiligo patients could enhance the systemic production of H_2O_2. The downstream antioxidant enzymes that neutralize H_2O_2 i.e. CAT and GPX showed significant decrease in their activities resulting in the generation of oxidative stress. Also significant decrease in the GSH levels and G6PDH activity contributes to the oxidative stress by preventing the non-enzymatic cycle to proceed to completion. GST and GR activities were not significantly affected in all age groups of vitiligo. Thus the present study shows that the disease affects individuals of any age group and systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients.

Evaluation of oxidative stress in different clinical types of vitiligo

Blood samples were collected from 124 patients with different clinical types of vitiligo (vulgaris, focal, acrofacial and segmental) and from 126 healthy controls for the analysis of the antioxidant status.

Significant increase in SOD activity and significant decrease in G6PDH was observed in all clinical types of vitiligo as compared to healthy controls. LPO, the index of oxidative stress was found to be significantly increased in all types of vitiligo. Catalase activity was found to be significantly decreased in vulgaris and focal types; however, no significant change was observed in segmental and acrofacial types of vitiligo. GPX activity was significantly lowered in vulgaris and focal types, while other types showed no significant change. No significant change was observed in GST and GR activities of all clinical types of vitiligo patients compared to controls. Whole blood GSH levels were significantly lowered in all types except focal vitiligo. No significant change was observed in vitamin E levels in any clinical type of vitiligo. This study suggests that the origin of oxidative stress is different in different clinical types of Gujarat vitiliginous patients.

Genetic association of T/C single nucleotide polymorphism in exon 9 of catalase gene in vitiligo patients of Gujarat and controls:

Our biochemical studies showed that systemic oxidative stress in vitiligo patients is due to the alteration in the antioxidant system in vitiligo patients compared to controls. Catalase and glutathione peroxidase activities were found to be significantly decreased in vitiligo patients compared to controls. We have attempted to explore whether reduced activity of these enzymes is linked to well documented single nucleotide polymorphisms present in the *catalase* and *glutathione peroxidase* genes.

One of the reported *catalase* single nucleotide polymorphisms in Caucasian population is a T/C silent substitution in *CAT* exon 9 (Asp-389) and a possible association was established between the T/C exon 9 *CAT* marker and vitiligo in Caucasian population (Casp et al 2002). However, there are no such studies in Indian population. In this study T/C exon 9 CAT marker was genotyped in vitiligo patients (n=140) and controls (n=143) of Gujarat by PCR – RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) method. Our results suggest that this polymorphism seems to be uninformative in Gujarat population.

Genetic association of glutathione peroxidase I codon 200 single nucleotide polymorphism in Gujarat vitiligo patients compared to controls:

Hu and Diamond (2003) reported that individuals with glutathione peroxidase I codon 200 variants exhibit compromised oxidative defense mechanism and reduced GPX 1 activity. They have shown that proline containing allele exhibits reduced GPX activity compared to leucine containing allele (Hu and Diamond, 2003). However, there are no reports on the association of *GPX* codon 200 polymorphism with vitiligo. Our studies on antioxidant status of vitiligo patients showed a significant decrease in the glutathione peroxidase activity in vitiligo patients compared to controls. An attempt was made to study the association of *GPX* codon 200 polymorphism

with vitiligo. Our results suggest that this polymorphism seems to be uninformative in Gujarat population.

Estimation of the protein levels of erythrocyte SODI in vitiligo patients compared to controls:

Our studies showed that there is significant increase in the SOD activity in vitiligo patients compared to controls. Western blot analysis was done to explore whether the increased SOD activity is due to the increased expression of the *SOD I* gene. SOD activity showed significant increase in vitiligo patients compared to controls. However the western blot and densitometric analysis showed that there is no significant change in SOD protein levels in vitiligo patients compared to controls.

Evaluation of autoimmune hypothesis in Gujarat vitiligo patients compared to Controls:

We have analyzed the sera of 102 vitiligo patients and 127 controls for the presence of antimelanocyte antibodies by ELISA. A significant increase in the antimelanocyte antibody levels was seen in vitiligo patients compared to controls. Sixty-six percent of vitiligo patients had antimelanocyte antibodies in their circulation. The levels of antimelanocyte antibodies were found to be significantly increased in vitiligo patients compared to controls. All the age groups showed significant increase in the antimelanocyte antibody levels in vitiligo patients compared to controls. Different clinical types of vitiligo patients also showed significant increase in the antibody levels compared to controls. No significant change was observed in the levels of antimelanocyte antibodies in active vitiligo compared to stable vitiligo patients. We have compared the antimelanocyte antibody levels in patients during the onset of vitiligo (< 3 months) with those suffering with vitiligo for a long duration (>3 months) to find whether autoimmunity is the cause in precipitating vitiligo in susceptible patients. Significant decrease ($p<0.005$) was observed in the levels

of antimelanocyte antibodies in <3 months patients compared to >3 months patients.

We have also performed western blot analysis of membrane antigens of SK Mel 28 cells to identify the pigment cell antigens defined by antibodies in the sera of vitiligo patients. One prominent protein band of approximately 200 kDa was obtained in vitiligo patients compared to controls.

Estimation of Acetylcholine esterase (evaluation of neurochemical hypothesis) in Gujarat vitiligo patients compared to controls:

The study consisted of 124 vitiligo patients and 126 age and sex matched controls. Acetylcholine esterase showed significant decrease in overall vitiligo patients compared to controls. Patients were classified into different clinical types for our further analysis. However there is no significant difference in the AChE activity in different clinical types. Active and stable vitiligo also did not show any significant difference. Further, AChE activity in vitiligo patients was analyzed in different age groups and the age group 16-25 yrs showed a significant decrease in AChE activity compared to controls. Other groups did not show any difference in the activity compared to controls. In order to study the role of AChE in the genetically predisposed vitiligo patients, we analyzed its role in patients with positive family history and negative family history; however, no significant difference was observed in AChE activity.

The above study shows that impairment in the systemic antioxidant system is observed in vitiligo patients compared to controls, which indicates that melanocyte damage in vitiligo may be linked with generalized oxidative stress. The disease affects individuals of any age group and systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients. However, the origin of oxidative stress may be different in different clinical types of vitiligo. Oxidative stress in vitiligo patients may cause the inactivation of acetylcholine esterase, which allows the accumulation of acetylcholine that

is toxic to melanocytes. Thus the impairment of acetylcholine esterase activity aggravates the disease. Our results suggest that there is a link between oxidative stress and neurochemical hypotheses in the pathogenesis of Gujarat vitiligo patients.

Our biochemical studies suggest that oxidative stress could as the trigger for precipitation of the disease in Gujarat vitiligo patients. However, our genetic studies suggest that *CAT* exon 9 T/C and glutathione peroxidase exon 2 C/T polymorphisms are not associated with Gujarat vitiligo patients suggesting for the presence of novel SNP/s in the *CAT and GPX* genes. Also our SOD 1 western results suggest that increased SOD activity in vitiligo patients is not due to increase in the SOD1 protein levels suggesting for the presence of novel SNP/s in the *SOD* gene, which could enhance the SOD 1 activity in Gujarat vitiligo patients.

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